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by Bryn L Adams

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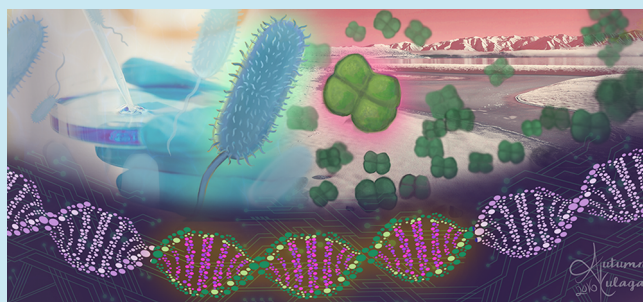
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14. ABSTRACT <i>Escherichia coli</i> (<i>E. coli</i>) has played a pivotal role in the development of genetics and molecular biology as scientific fields. It is therefore not surprising that synthetic biology (SB) was built upon <i>E. coli</i> and continues to dominate the field. However, scientific capabilities have advanced from simple gene mutations to the insertion of rationally designed, complex synthetic circuits and creation of entirely synthetic genomes. The point is rapidly approaching where <i>E. coli</i> is no longer an adequate host for the increasingly sophisticated genetic designs of SB. It is time to develop the next generation of SB chassis; robust organisms that can provide the advanced physiology novel synthetic circuits will require to move SB from the laboratory into fieldable technologies. This can be accomplished by developing chassis-specific genetic toolkits that are as extensive as those for <i>E. coli</i> . However, the holy grail of SB would be the development of a universal toolkit that can be ported into any chassis. This viewpoint article underscores the need for new bacterial chassis, as well as discusses some of the important considerations in their selection. It also highlights a few examples of robust, tractable bacterial species that can meet the demands of tomorrow's state-of-the-art in SB. Significant advances have been made in the first 15 years since this field has emerged. However, the advances over the next 15 years will occur not in laboratory organisms, but in fieldable species where the potential of SB can be fully realized in game changing technology.					
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The Next Generation of Synthetic Biology Chassis: Moving Synthetic Biology from the Laboratory to the Field

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ABSTRACT: *Escherichia coli* (*E. coli*) has played a pivotal role in the development of genetics and molecular biology as scientific fields. It is therefore not surprising that synthetic biology (SB) was built upon *E. coli* and continues to dominate the field. However, scientific capabilities have advanced from simple gene mutations to the insertion of rationally designed, complex synthetic circuits and creation of entirely synthetic genomes. The point is rapidly approaching where *E. coli* is no longer an adequate host for the increasingly sophisticated genetic designs of SB. It is time to develop the next generation of SB chassis; robust organisms that can provide the advanced physiology novel synthetic circuits will require to move SB from the laboratory into fieldable technologies. This can be accomplished by developing chassis-specific genetic toolkits that are as extensive as those for *E. coli*. However, the holy grail of SB would be the development of a universal toolkit that can be ported into any chassis. This viewpoint article underscores the need for new bacterial chassis, as well as discusses some of the important considerations in their selection. It also highlights a few examples of robust, tractable bacterial species that can meet the demands of tomorrow's state-of-the-art in SB. Significant advances have been made in the first 15 years since this field has emerged. However, the advances over the next 15 years will occur not in laboratory organisms, but in fieldable species where the potential of SB can be fully realized in game changing technology.



In the past 15 years, synthetic biology (SB) has emerged as an interdisciplinary field that applies engineering approaches to the genetic components of natural systems to generate novel, designed biological networks. A number of tools have been developed to rationally design, synthesize, and build genetic networks in a modular fashion for customized biological functions. Although vast research efforts have focused on the programming aspect, far less have focused on the chassis—or host organism itself. In fact, it can easily be argued that SB was built upon a very small set of domesticated, laboratory organisms, primarily utilizing *Escherichia coli* (*E. coli*) and *Saccharomyces cerevisiae*. These organisms were pervasive in molecular biology and genetic engineering because they were highly adapted to laboratory conditions, where rapid growth rates and abundant protein production were valued, and became legacy SB chassis as this field grew out of molecular biology and genetic engineering. However, it is becoming increasingly clear that these are not the ideal chassis and new chassis are required for SB capabilities to advance in medicine, academia, industry, and government. This viewpoint article focuses on the need for new SB chassis bacteria, particularly for use in biotechnology applications relevant to fieldable technologies, and highlights some potential organisms and toolkit development for the next generation SB chassis.

In classical engineering terms, a chassis is the framework or foundation that supports other physical components for an engineered structure. In SB, a chassis refers to the organism that serves as a foundation to physically house genetic

components and supports them by providing the resources to function, such as transcription and translation machinery. Undoubtedly, *E. coli* is the most commonly used chassis in SB, with the largest available toolkit of computational design programs, genetic parts and regulatory elements (promoters, ribosomal binding sites (RBS), and terminators), as well as DNA vectors, and DNA delivery protocols. Complex pathways have been programmed into *E. coli* using these extensive toolkits that impart sophisticated functions to these cells. For example, Roquet *et al.*¹ programmed *E. coli* to remember three different inputs, in order, and respond accordingly. Their approach was scalable, enabling environmental biosensors that can log and respond to a complex series of events. New bacterial chassis are required to capitalize on the advanced functions and applications and expand the potential of SB further. Unfortunately, moving to new chassis bacteria is not as simple as porting the toolkits and circuits developed in *E. coli* into another bacterial species. The genetic expression and regulation of synthetic circuits are highly host-specific. DNA binding affinity of homologous RNA polymerases and transcription factors are not well conserved, so circuitry with high functionality in *E. coli* does not translate well to another, even related species. To move SB from laboratory demonstrators to fieldable technology, it is imperative that a panel of robust chassis be created. Each new chassis will require a

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comprehensive toolkit equal to *E. coli*, or alternatively, a large toolkit of universal components with a high degree of functionality in any bacterial species.

Bacteria have evolved a wide range of useful physiological properties that can be leveraged for government, industry and general biotechnology applications. Although biosynthesis or biotransformation capabilities are valuable characteristics of the next generation of SB chassis in terms of fieldable technology, it is more important to operate under extreme conditions. This includes extreme temperatures and pH, high osmotic pressures, low resource availability, as well as the ability to outcompete other microbes. Developing a panel of chassis organisms that can thrive under a variety of conditions is critical for expanding the current scope of SB. It will make it possible for engineered organisms to be integrated into fieldable technology for the first time. For example, the record and respond module could be introduced into a new chassis adept to operating in a biohybrid device and provide the advanced biological sense-record-respond capabilities. Regardless of the species selected or function performed, there are several key considerations in the development of new SB chassis (Figure 1). First, a large

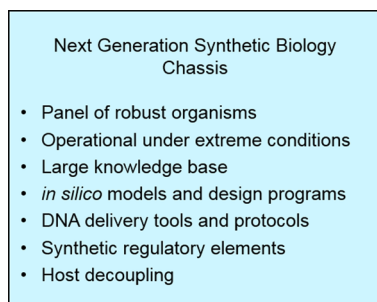


Figure 1. Considerations for the development of the next generation of synthetic biology chassis.

knowledge base of the chassis needs to be established to facilitate development of accurate *in silico* models that will aid in the design of next generation chassis synthetic circuits. Current models for *E. coli* still strive to accurately capture the *in vivo* complexity of synthetic circuits,² so considerable effort will be required to establish models for other bacterial species. Chassis optimized transcription and translational control elements are also needed to tightly control gene expression and chassis specific programming languages should be developed to rapidly design complex circuits. Currently, Chris Voigt and colleagues at Massachusetts Institute of Technology (MIT) are working to extend the bacterial programming language Cello beyond *E. coli* to function in other organisms, such as *Bacteroides* and *Pseudomonas*. Additionally, tools are needed to introduce the designed genetic circuits into chassis that are not naturally competent in DNA uptake. Traditional approaches include conjugation and electroporation, as well as use of chemically competent cells and protoplasts, but more efficient and universal methodologies, including high throughput transformation, are needed. Once the novel circuit is transformed into the chassis, these circuits will inevitably compete with the native host system for resources, such as energy sources and DNA replication, transcription, and translation elements. As a result, it may be necessary to uncouple the synthetic system from the host by designing the synthetic circuits to operate independently or streamline/minimize the host genome to provide more resources for the circuit. Some of these

considerations have already begun to be addressed in robust bacterial species utilized in metabolic engineering for industrial purposes, including *Pseudomonas putida* (*P. putida*) and *Bacillus subtilis* (*B. subtilis*). *Geobacillus* has also garnered attention as a next generation chassis because it is a spore forming thermophile. Other possible bacteria that may prove useful as government and industry chassis include photosynthetic cyanobacteria and the highly stress-resistant *Deinococcus*. Both organisms have unique physiological properties desirable for a chassis, but will likely require more extensive toolkit development than *P. putida* or *B. subtilis*.

Pseudomonas putida is an ideal member of the next generation SB chassis panel as it is a common Gram negative soil bacterium, certified as “generally recognized as safe” (GRAS). This organism has also been certified as a Host Vector Biosafety (HVB) strain and approved for release into the environment. Beyond environmentally safe, *P. putida* meets many of the criteria for a next generation SB chassis because it naturally thrives in harsh physiochemical conditions and can adapt to rapidly changing conditions, including high temperature, extreme pH, toxins, solvents, oxidative stress, and osmotic perturbation. This organism also has low nutritional requirements and a highly versatile metabolism, allowing energy to be derived from a number of sources.³ *P. putida* has the inherent cellular machinery to survive and thrive in any niche. As a result of being an environmental bacterial model and the laboratory workhorse for bioremediation, the type strain is a well-established host for cloning and gene expression and has genome-scale models available for *in silico* studies. Stable cloning has been extensively demonstrated, specifically by the use of Tn5-derived mini-transposon system for DNA integration into the genome.⁴ Progress has recently been made in the development of genetic tools to tune synthetic circuit expression. Elmore *et al.*⁵ has developed a genome integration and reporter system using serine integrases and identified synthetic *P. putida* promoters from a library with a wide variety of expression levels. The techniques used in promoter development are extensible to the development of other tools, including synthetic terminator discovery or for rapid integration of synthetic pathways.

Similar to *P. putida*, the Gram positive bacterium *B. subtilis* is a long-standing model organism and industrial workhorse due to its endogenous secretory pathways for enzyme and antibiotic production. This nonpathogenic organism has been certified as GRAS and used extensively in biotechnology applications, but its use in SB has been comparatively limited.⁶ Physiological features that make *B. subtilis* an attractive SB chassis include natural competence, easy DNA integration into chromosome, and a wide range of natural two-component systems and quorum-sensing systems that can be modified for biosensor applications. Stable cloning has been extensively demonstrated in *B. subtilis* using expression vectors for protein production and integrative vectors to construct gene knockouts and fusions. Recently, a collection of *B. subtilis* genetic components has been published, including constitutive and inducible promoters and epitope tags;⁷ however, tenability has been limited. Guiziou *et al.*⁶ engineered a toolkit of additional promoters, RBS, and proteolysis tags to control gene expression at the transcriptional, translational, and protein levels. CRISPR-Cas9 has also been investigated for site-specific mutations, gene insertions, and to enable continuous gene editing.⁸ Additionally, there are a number of bioinformatics and computational tools available to aid in synthetic circuit design.

Geobacillus has great chassis potential as it is one of the more tractable extremophiles, although its genetic toolkit development is lesser compared to *P. putida* and *B. subtilis*. This Gram positive thermophile includes spore forming aerobic and facultative anaerobic species. With optimal growth temperatures between 45 and 70 °C, they may prove to be useful chassis for high temperature operational conditions. This genus is currently used by industry for its catabolic versatility and its ability to secrete commercially useful enzymes. Although there are a few plasmids and shuttle vectors developed specifically for *Geobacillus*, tools for the closely related *B. subtilis* can also be used. DNA can be introduced into host cells *via* protoplasts, electroporation, or conjugation. However, selection markers are limited due to its high growth temperatures. Integration cassettes for chromosomal integration are available, as are reporter genes and a limited number of constitutive and inducible promoters.⁹

Nutrient limitation is a persistent challenge for the integration of a SB chassis into fieldable technology. Cyanobacteria are an attractive chassis because of their photosynthetic ability, tractable genetics, and fast growth. The toolkit components for cyanobacteria are limited, but do include integrative and replicative vectors, natural and synthetic promoters, high efficiency RBS, and terminators. Furthermore, the CRISPR-Cas system has been investigated for one cyanobacteria species,¹⁰ which is important for the development of this group as a chassis. Another key feature of a chassis for fieldable technology is the ability to withstand many extreme stresses. The bacterial genus *Deinococcus* is well-known for its resistance to ionizing radiation, however it also shows remarkable resistance to desiccation, UV radiation and oxidizing agents, primarily due to its capacity for rapid DNA repair. *Deinococcus* species have been isolated from extreme environments including air dust, desert soil, and cold environments and requires only minimal media for growth. This highly robust organism is an ideal chassis because genetic toolkits have begun to be established for some species. Components include a variety of shuttle vectors, native promoters and repressors. Unfortunately, the shuttle vectors tend to be quite large and species specific. Chromosomal mutations in *Deinococcus* have been well documented, with insertions and deletions created using homologous recombination of nonreplicative plasmids. Although transformation efficiencies tend to be low, exogenous DNA has been introduced using chemically competent cells and electroporation.¹¹

Building genetic toolkits for each member of the next generation SB chassis panel is an extensive and laborious undertaking. For this reason, the holy grail of SB remains a synthetic system that is universal and can be transformed into and operate efficiently within any chassis. Kushwaha *et al.*¹² has recently made progress toward this goal by developing Universal Bacterial Expression Resource (UBER). Genetic circuits and metabolic pathways were engineered in Gram negative and positive organisms using an autonomously regulated T7 RNA polymerase expression system and host-independent promoters. Although this work overcomes several key challenges in establishing a universal synthetic toolkit, such as T7 RNA polymerase toxicity, many challenges remain. These include a wholly host-independent set of regulatory element that can rapidly adapt to unique host transcription and translation machinery or deliver its own, as well as a universal mechanism for delivering exogenous DNA and integrating it

into the chromosome. Regardless of whether a panel of novel chassis organisms with corresponding complete toolkits, or the ultimate prize of an absolute universal synthetic toolkit is pursued, SB must part ways with *E. coli*. Instead, it must embrace more robust natural species, or even synthetic cells, in order to move out of the laboratory and into fieldable systems.

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Notes

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